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Clonal Species *Trichoderma parareesei* sp. nov. Likely Resembles the Ancestor of the Cellulase Producer *Hypocrea jecorina*/*T. reesei*▽†

Lea Atanasova,[1]‡ Walter M. Jaklitsch,[2]‡ Monika Komoń-Zelazowska,[1] Christian P. Kubicek,[1] and Irina S. Druzhinina[1]*

Research Area of Gene Technology and Applied Biochemistry, Institute of Chemical Engineering, Vienna University of Technology, Getreidemarkt 9/1665, A-1060 Vienna, Austria,[1] and Faculty Centre of Biodiversity, University of Vienna, Rennweg 14, A-1030 Vienna, Austria[2]

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We have previously reported that the prominent industrial enzyme producer *Trichoderma reesei* (teleomorph *Hypocrea jecorina*; Hypocreales, Ascomycota, Dikarya) has a genetically isolated, sympatric sister species devoid of sexual reproduction and which is constituted by the majority of anamorphic strains previously attributed to *H. jecorina*/*T. reesei*. In this paper we present the formal taxonomic description of this new species, *T. parareesei*, complemented by multivariate phenotype profiling and molecular evolutionary examination. A phylogenetic analysis of relatively conserved loci, such as coding fragments of the RNA polymerase B subunit II (*rpb2*) and GH18 chitinase (*chi18-5*), showed that *T. parareesei* is genetically invariable and likely resembles the ancestor which gave raise to *H. jecorina*. This and the fact that at least one mating type gene of *T. parareesei* has previously been found to be essentially altered compared to the sequence of *H. jecorina*/*T. reesei* indicate that divergence probably occurred due to the impaired functionality of the mating system in the hypothetical ancestor of both species. In contrast, we show that the sexually reproducing and correspondingly more polymorphic *H. jecorina*/*T. reesei* is essentially evolutionarily derived. Phenotype microarray analyses performed at seven temperature regimens support our previous speculations that *T. parareesei* possesses a relatively high opportunistic potential, which probably ensured the survival of this species in ancient and sustainable environment such as tropical forests.

Trichoderma reesei, the anamorph of the pantropical saprotrophic ascomycete *Hypocrea jecorina*, is used in the biotechnological industry for the production of cellulolytic and hemicellulolytic enzymes and recombinant proteins (13, 21). Accordingly, strong interest in this fungus has also recently reemerged in attempts to produce second-generation biofuels to reduce carbon dioxide emissions and the dependence on fossil fuels (oil) (15, 17).

Trichoderma reesei was originally collected on the Solomon Islands during World War II, where it destroyed canvas and other cellulose-containing materials of the U.S. army (18). It is unique among industrial fungi, as *T. reesei* was known only from the single isolate QM 6a for 50 years, and all genetically improved mutant strains used in biotechnology today have been derived from it. Kuhls et al. (14) found that *T. reesei* was indistinguishable from mycelial cultures of the pantropical ascomycete *Hypocrea jecorina*, on the basis of sequences of the internal transcribed spacer of the rRNA gene cluster and randomly amplified polymorphic DNA (RAPD) fingerprinting analysis. Thus, they established the anamorph-teleomorph connection, which is still valid. Other putative anamorphs of *H. jecorina* have more recently been identified as frequent inhabitants of soils in tropical forests of Southeast Asia, South America, and the South Pacific region (1, 7, 12). Druzhinina et al. (4), however, have recently shown that the majority of anamorphic strains are genetically isolated from *H. jecorina*/*T. reesei* and form at least two new phylogenetic species. The more frequent one (*T. parareesei* nom. prov. [4]) was shown to be closely related to and also sympatric with *H. jecorina* but exclusively asexual (clonal or agamospecies). In that work we also provided some details on the ecophysiological characteristics of *H. jecorina* and its sister species. We showed that the extremely efficient cellulase-producing strains are present in all these phylogenetic species and that in general their carbon metabolisms are very similar, although the clonal species are more versatile and efficient in the utilization of their preferred substrata. No details on carbon utilization profiles or temperature-dependent growth rates have been presented. Striking differences between *H. jecorina*/*T. reesei* and *T. parareesei* in conidiation intensity, photosensitivity, and mycoparasitism have been found, all suggesting that the latter species occupies a separate ecological niche and has much stronger opportunistic potential than *H. jecorina* (4).

Here we provide the formal taxonomic description of *Trichoderma parareesei* on the basis of macro- and micromorphologies, carbon utilization profiles at different temperatures, and phylogenetic analysis. We show that this species likely resembles the ancestor of *H. jecorina*/*T. reesei*.

MATERIALS AND METHODS

Material studied. The strains used in this work, their origin, and the NCBI GenBank sequence accession numbers are listed in Table 1. The isolates are stored at −80°C in 50% glycerol in the Collection of Industrial Microorganisms of Vienna University of Technology (TUCIM). For convenience, TUCIM num-

* Corresponding author. Mailing address: Research Area of Gene Technology and Applied Biochemistry, Institute of Chemical Engineering, Vienna University of Technology, Getreidemarkt 9/1665, A-1060 Vienna, Austria. Phone: 4315880117202. Fax: 4315880117299. E-mail: druzhini@mail.zserv.tuwien.ac.at.

‡ These two authors contributed equally to the manuscript and are listed in alphabetical order.

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TABLE 1. Strains of *T. parareesei* and *H. jecorina*/*T. reesei* as well as other species from *Trichoderma* section *Longibrachiatum* used in this study[a]

Taxon	C.P.K. strain no.	Other strain no.	Origin	GenBank accession no.	
				chi18-5	*rpb2*
Reesei subclade					
Trichoderma parareesei sp. nov.	717 type	CBS 125925, TUB F-1066	Mexico	HM182987	HM182963
	3426	G.J.S. 07-26	Ghana	HM182991	HM182966
	661	CBS 125862, TUB F-728	Argentina	HM182990	HM182965
	665	TUB F-733	Argentina	HM182992	HM182967
	3420	G.J.S. 04-41	Brazil	HM182989	HM182964
	634	TUB F-730	Sri Lanka	HM182993	HM182968
	3692		Ethiopia	HM182987	HM182962
Hypocrea jecorina	160	G.J.S. 85-236	Celebes, Indonesia	HM183002	HM182977
	282	G.J.S. 97-177	French Guiana	HM182999	HM182974
	1392	G.J.S. 86-401	Puerto Rico	HM183005	HM182980
	1282	G.J.S. 85-249	Celebes, Indonesia	HM182998	HM182973
	158	G.J.S. 85-229	Celebes, Indonesia	HM183003	HM182978
	159	G.J.S. 85-230	Celebes, Indonesia	HM183004	HM182979
	1127	G.J.S. 93-23	New Caledonia	HM183000	HM182975
	1337	G.J.S. 93-22	New Caledonia	HM183001	HM182976
	917	CBS 383.78, QM 6a	Solomon Islands	HM182994	HM182969
	3418	G.J.S. 06-138	Cameroon	HM182997	HM182972
	3419	G.J.S. 06-140	Cameroon	HM182996	HM182971
	155	G.J.S. 86-404	Brazil	HM182995	HM182970
Other species from *Trichoderma* section *Longibrachiatum*					
Trichoderma sp. strain C.P.K. 524	523	TUB F-1034	Taiwan	HM183006	HM182981
	524	TUB F-1038	Taiwan	HM183007	HM182982
Trichoderma sp. strain C.P.K. 1837	1837		Ethiopia	HM183011	HM182986
T. longibrachiatum	1254	CBS 48978	Colombia	DQ087242[b]	EU401511[b]
T. saturnisporum	1266	CBS 33070, ATCC 18903	Georgia, USA	HM183009	HM182984
H. schweinitzii	2002	CBS 121275	Germany, Europe	HM183008	HM182983
T. pseudokoningii	1277	G.J.S. 81-300		HM183010	HM182985

[a] Strains of *T. parareesei* sp. nov., *H. jecorina*, and *Trichoderma* sp. strain C.P.K. 524 were published earlier (1, 4, 12). ATCC, American Type Culture Collection; CBS, Centralbureau voor Schimmelcultures; G.J.S., collection of Gary J. Samuels, USDA, Beltsville, MD; TUB, collection of George Szakacs, TU Budapest, Hungary.
[b] Previously published by Jaklitsch et al. (8).

bers (CPK) are used for the strains throughout the article; in addition, numbers of the original isolators' collections are listed in Table 1.

DNA extraction, PCR amplification, and sequencing. Mycelia were harvested after 2 to 4 days of growth on 3% malt extract agar (MEA; Merck, Germany) at 25°C, and genomic DNA was isolated using a Qiagen (Hilden, Germany) DNeasy plant minikit following the manufacturer's protocol. Amplification of fragments of *chi18-5* (GH18 chitinase CHI18-5, previously *ech42*) and *rpb2* (RNA polymerase subunit II ß) was performed as described previously (9). PCR fragments were purified (PCR purification kit; Qiagen) and sequenced at Eurofins MWG Operon (Ebersberg, Germany).

Phylogenetic analysis. Phylogenetic analysis was done essentially as described previously (4, 9). Briefly, DNA sequences were aligned by use of the Clustal X program (version 1.81) and visually checked in GeneDoc software (version 2.6). The possibility of intragenic recombination, which would prohibit the use of each locus for phylogenetic analysis, was tested by linkage disequilibrium-based statistics implemented in DnaSP software (version 4.50.3) (19). The neutral evolution was tested by Tajima's test, implemented in the same software. The interleaved NEXUS file was formatted using the PAUP* program (version 44.0b10) (22). As the sample size is relatively small (1,670 characters per 26 sequences for the biggest data set), the unconstrained GTR+I+G substitution model was applied to all sequence fragments (Table 2). Metropolis-coupled Markov chain Monte Carlo (MCMC) sampling was performed using the MrBayes program (version 3.0B4) with two simultaneous runs of four incrementally heated chains for 1 million generations. Bayesian posterior probabilities (PPs) were obtained from the 50% majority rule consensus of trees sampled every 100 generations after removal of the first trees using the "burnin" command. The number of discarded generations was determined for each run on the basis of visual analysis of the plot showing the generation versus the log probability of observing the data. PP values lower than 0.95 were not considered significant. Summaries of the model parameters and nucleotide characteristics of the loci used are given in the Table 2.

Phenotype microarrays. Growth rates on different carbon sources were analyzed using a phenotype microarray system for filamentous fungi (Biolog Inc., Hayward, CA), as described by Druzhinina et al. (2) and Friedl et al. (5). Briefly, strains were cultivated on 3% MEA for 5 days. Conidial inocula were prepared by rolling a sterile, wetted cotton swab over sporulating areas of the plates. The conidia were then suspended in sterile Biolog FF inoculating fluid (0.25% Phytagel, 0.03% Tween 40), gently mixed, and adjusted to a transmission of 75% at 590 nm (using a Biolog standard turbidimeter calibrated to the Biolog standard for filamentous fungi). A total of 90 μl of the conidial suspension was dispensed into each of the wells of the Biolog FF microplates (Biolog Inc.), which were incubated at 15, 20, 25, 30, 35, 40, and 45°C in darkness. The optical density (OD) at 750 nm (for detection of mycelial growth [2, 5]) was measured after 18, 24, 42, 48, 66, 72, and 96 h using a microplate reader (Biolog Inc.). Statistical analyses were performed using the Statistica software package (version 6.1; StatSoft Inc., Tulsa, OK).

Growth rate determination on agar plates and morphological observations. The micromorphology of conidiation structures, growth rates, and the comparative morphology of cultures were determined and morphological terms were applied as described by Jaklitsch (10). Growth rates at 15, 25, 30, and 37°C were recorded in a single experiment using 2 strains each of *T. parareesei* and *T. reesei*. In addition, images of cultures grown on 3% MEA for 5 days at 25°C under alternating 12 h of light/12 h of dark were taken. Color was determined as described by Kornerup and Wanscher (11).

Phytotoxicity assays. Phytotoxicity assays were carried out using seeds of garden cress (*Lepidium sativum*) from Austrosaat AG (Vienna, Austria) and strains CBS 125925, CBS 125862, and C.P.K. 665 of *T. parareesei* and strains C.P.K. 917 (QM 6a), C.P.K. 1282, and C.P.K. 3419 of *H. jecorina.* Samples without *Trichoderma* were used as controls. The assay was designed on the basis of a 24-well plate, where each well was inoculated with one seed of *L. sativum*. Surface sterilization of the seeds was performed in 96% ethanol for 15 min, followed by washing of the seeds with sterile double-distilled water, with this step

TABLE 2. Nucleotide properties of phylogenetic markers and MCMC parameters[b]

Parameter	Phylogenetic marker		
	rpb2	*chi18-5*	Concatenated data set
Fragment characterization	Partial exon	Partial exon	Not applicable
No. of characters	852	818	1,670
No. parsimony informative	80	88	168
No. constant	685	680	1,365
Parameters of MCMC analysis			
Mean nucleotide frequency[a] (A/C/G/T)	0.24/0.28/0.28/0.20	0.23/0.33/0.24/0.20	0.24/0.30/026/0.20
Substitution rates[a]			
A↔C	0.12	0.10	0.11
A↔G	0.23	0.23	0.25
A↔T	0.03	0.12	0.06
C↔G	0.08	0.10	0.09
C↔T	0.44	0.37	0.39
G↔T	0.09	0.06	0.08
Alpha[a]	0.18	21.32	0.23
No. of MCMC generations (10^6)/no. of runs	1/2	1/2	2/2
PSRF[a]	1.00	1.00	1
No. of chains/temp (λ)	4/0.2	4/0.2	4/0.2
Sampling frequency	100	100	100
No. of discarded first generations	200	300	300
Total tree length (substitutions/site)	0.58	0.3	0.38

[a] Alpha (shape parameter of the gamma distribution) and potential scale reduction factor (PSRF) values were estimated after GTR MCMC sampling and burning.
[b] Twenty-nine sequences in total were analyzed.

being repeated twice. Individual seeds were introduced into 20 of the 24 wells filled with synthetic low nutrition agar (SNA) medium (for 1 liter, 1 g of KH_2PO_4 and KNO_3, 0.5 g $MgSO_4 \cdot 7H_2O$ and KCl, 0.2 g glucose and sucrose, and 20 g of agar-agar; Merck, Darmstadt, Germany), leaving 4 wells for the control of the fungal growth. Pregrown mycelium was introduced to 20 seedlings per strain. The plates were incubated at 25°C under a rhythmic illumination cycle (12 h of light/12 h of dark) for 8 days. Afterwards, the plants were inspected under a stereomicroscope and the lengths of their stems were measured.

MycoBank accession number. The sequence of *Trichoderma parareesei* Jaklitsch, Druzhinina & Atanasova, sp. nov., has been deposited in MycoBank under accession no. MB 515503 (Fig. 1).

RESULTS

Taxonomy. Differt a Trichodermate reesei absentia teleomorphosis, sporulatione multo magis abundante, conidiophoribus sinuosis et conidibus parvioribus, uniformioribus in forma et magnitudine. Phialides in agaro CMD lageniformes vel ampulliformes, 4.5 ad 11 μm longae, 2.5 ad 3.8 μm latae. Conidia viridia, ellipsoidea vel oblonga, glabra, 3.3 ad 6.2 μm longa, 2.5 ad 3.5 μm lata.

Morphology and growth rates of *T. parareesei*. Table 3 presents some characteristics of *T. parareesei*. The morphology and growth rates of *T. parareesei* follow. Optimum growth at 30 to 37°C on all media. On CMD (Sigma corn meal agar plus 10% dextrose) mycelium covering the plate within 3 days at 25 to 37°C. Colony on CMD at 25 to 37°C hyaline, thin; mycelium dense, not zonate, concentrated on the agar surface, of thick, radially arranged primary hyphae and numerous delicate secondary hyphae forming a reticulum. Hyphal width decreasing with increasing temperature. Aerial hyphae scant, short, becoming fertile. Autolytic activity and coilings lacking or inconspicuous. No diffusing pigment produced or agar turning slightly yellowish, 1B3 to 3B3; no distinct odor produced. Chlamydospores at 30 to 37°C after 1 week uncommon, slightly increased in number relative to that at lower temperatures, terminal, mostly in thin, 3- to 4-μm-wide hyphae, less commonly intercalary in wider hyphae, 6 to 22 μm long by 4 to 16 μm wide, length/width ratios of 0.9 to 2.6 ($n = 33$), (sub)globose to pyriform, less commonly fusoid or oblong, smooth, sometimes 2 celled. Conidiation starting after 1 day on simple conidiophores of a straight, flexible main axis often sterile at the tip, and several tree-like side branches, paired, unpaired, or radially emergent from the main axis; appearing as minute white shrubs 0.3 to 0.6 mm in diameter, concentrated in proximal areas and in one to several diffuse, at higher temperatures often narrower, concentric zones. Shrubs loosely disposed and firmly attached to surface hyphae; erect, first with straight to sinuous sterile elongations, becoming green and entirely fertile; terminally 2.5 to 3.5 μm wide, downwards 4.5 to 5 μm. Consecutively, after ca. 3 days, dark green pustules to nearly 2 mm in diameter formed, more or less arranged in concentric zones. Pustules loose, transparent, first white with sterile ends or elongations, turning pale to dark green, maturing from inside, ends becoming fertile; consisting of a loose reticulum bearing several straight or sinuous main axes with highly variable, straight or distinctly sinuous side branches. Conidiophores (all branches) generally narrow, 2.0 to 4.5 μm wide, thick walled, and with thickenings to 5.5 to 6.5 μm when old, flexuous, loosely arranged, usually unpaired, in right angles or inclined upwards, holding solitary phialides in right angles along their length, directly, or terminally on a supporting cell often similar in shape (intercalary phialides) or on few-celled cylindrical branches, sometimes in whorls of 3 on intercalary

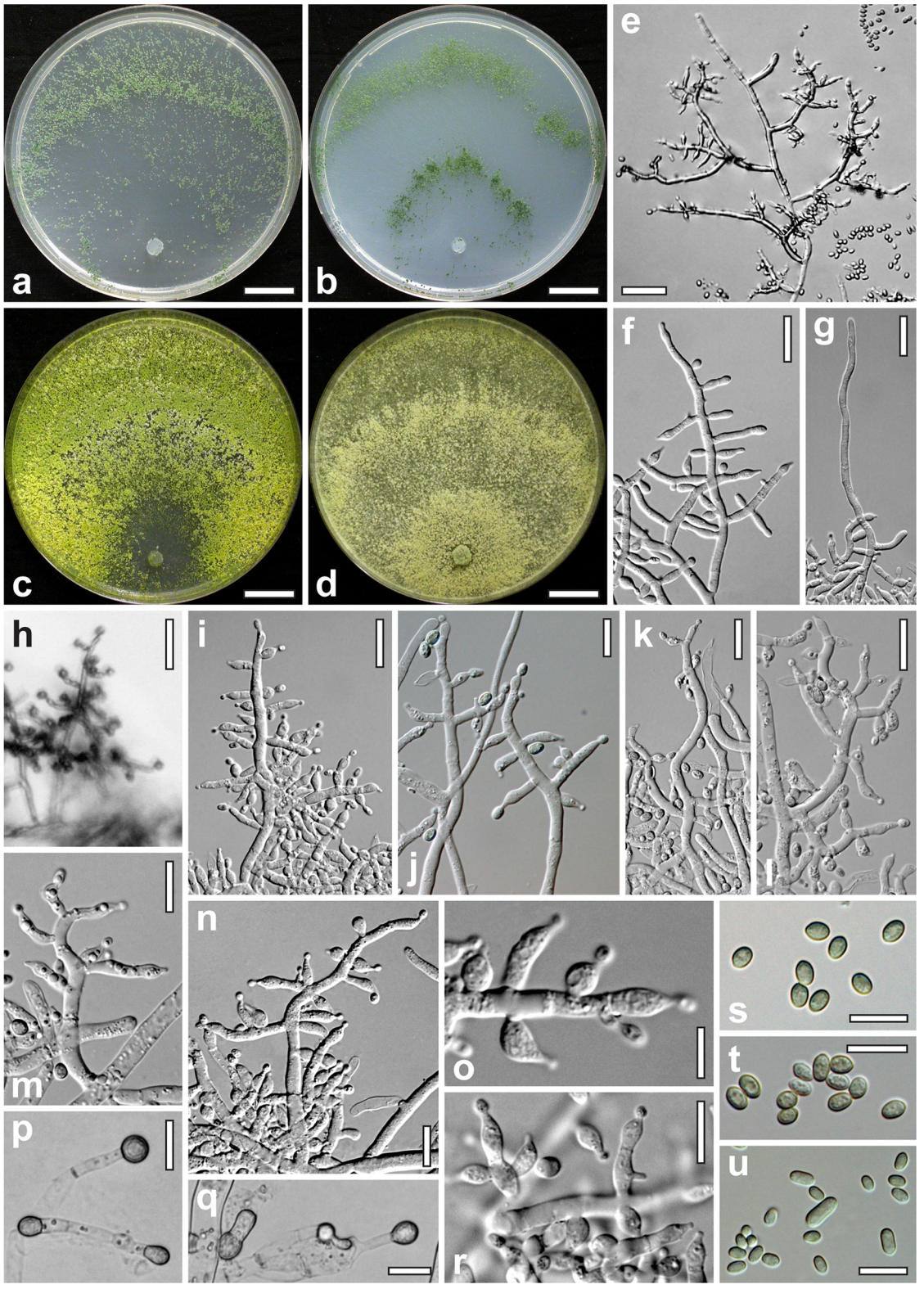

FIG. 1. *Trichoderma parareesei*. (a to c) Cultures after 4 days on CMD (a), SNA (b) and PDA (c); (e) conidiophore without cover glass (9 days); (f) young conidiophore (3 days); (g) young conidiophore with sterile elongation (3 days); (h) conidiophore of a shrub on growth plate (2 days); (i to n) conidiophores after 3 days (i and n) and after 9 days (j to m); (o and r) phialides at 2 days (o) and 8 days (r); (p and q) chlamydospores (7 days); (s and t) conidia (8 days); (e to t) growth on CMD; (a to c, p, and q) growth at 30°C; (e and j to m) growth at 15°C; (f to i, n, o, and r to t) growth at 25°C; (a to c, f, g, i, and n to t) strain C.P.K. 717; (e, h, and j to m) strain C.P.K. 661; (d and u) *Hypocrea jecorina* (*Trichoderma reesei*); (d) culture on PDA after 4 days at 30°C (C.P.K. 917); (u) conidia (C.P.K. 1127, SNA, 25°C, 4 days). Scale bars: 15 mm (a to d), 30 μm (e and h), 15 μm (f, g, i, k, l, p, and q), 10 μm (j, m, n, and r to u), and 5 μm (o).

TABLE 3. Colony phenotype and anamorph characteristic of the species discussed

Parameter	*T. parareesei* sp. nov.	*H. jecorina*/*T. reesei*
Colony type on CMD	Conidia formed abundantly in shrubs and well-defined pustules	Only shrubs; no well-defined pustules formed
Colony radius (mm) at 48 h		
CMD		
15°C	19–21	8–19
25°C	44–47	34–44
30°C	50–56	44–55
37°C	50–53	37–58
PDA		
15°C	15–16	9–16
25°C	44–48	36–49
30°C	53–56	45–59
37°C	57–61	42–59
SNA		
15°C	17–18	9–15
25°C	40–43	32–40
30°C	50–55	40–60
37°C	53–58	32–64
Conidia	Uniformly ellipsoidal; more variable, ellipsoidal to cylindrical at 37°C	Variable, ellipsoidal or oblong with parallel sides
Conidium		
Length (μm)	3.3–6.2 (3.8–4.5)[a]	3.5–9.0 (3.5–6.0)
Width at widest point (μm)	2.5–3.5 (2.8–3.2)	2.2–4.0 (2.5–3.3)
Length/width	1.2–2.0[b] (1.3–1.5)	1.2–2.7[c] (1.3–1.9)
Phialides	Lageniform or ampulliform, often with a cylindrical neck, usually with distinct widening at or above the middle	Lageniform
Phialide		
Length (μm)	4.5–11 (5–8)	5.0–14.5 (6–10)
Width at widest point (μm)	2.5–3.8 (2.7–3.5)	2.2–4.0 (2.5–3.5)
Width at base (μm)	1.4–3.2 (1.7–2.4)	1.3–3.0 (1.7–2.5)
Length/width	1.3–3.6 (1.6–2.7)	1.5–4.6 (2–3.4)

[a] Data in parentheses represent the narrower ranges determined by the mean plus minus standard deviation.
[b] $n = 70$.
[c] $n = 75$.

phialides, but not originating at the same position. Phialides lageniform or ampulliform with often cylindrical neck, straight and symmetric or inequilateral and distinctly curved, with wide or constricted base, usually with distinct widening at or above the middle. Conidia formed in minute, mostly dry heads 10 μm in diameter. Conidia green, uniformly ellipsoidal, smooth, thick walled, eguttulate or with some minute guttules; scar indistinct or pointed; shape and size more variable, ellipsoidal to cylindrical at 37°C. On potato dextrose agar (PDA), mycelium covering the plate within 3 days at 25 to 37°C. Secondary hyphae forming a dense reticulum. Autolytic excretions conspicuous at the colony margin, positively correlated with increasing temperature. Conidiation on PDA and MEA conspicuously abundant, in numerous densely arranged and variably superposed bright yellow-green to dark green pustules (for cultures of *T. parareesei* and *H. jecorina* on 3% MEA, see Fig. S1 in the supplemental material). Diffusing pigment variable, yellow if present. On SNA, mycelium covering the plate within 3 days at 25 to 37°C. Colony similar to CMD; conidiation more abundant, denser, and more regularly arranged in concentric zones.

Holotype. The holotype was isolated from soil of a subtropical rain forest near Iguazu Falls, Argentina, on 4 September 1997, and is deposited as a dry culture (WU 30015; living cultures CBS 125925, C.P.K. 717, TUB F-1066).

Additional isolates examined were CBS 125862 (C.P.K. 661, TUB F-728) from subtropical rain forest, near Iguazu Falls, Argentina, 4 September 1997.

Habitat and distribution. *T. parareesei* has been isolated from soils of subtropical and tropical areas in South America (Brazil, Argentina, Colombia), Central America (Mexico), Africa (Ghana and Ethiopia), and India (4). Hojos-Carvajal et al. (7) noted that the fungus occurs in soil of the African oil palm (*Elaeis guineensis*) in South America.

Anamorph morphology of *Hypocrea jecorina*/*Trichoderma reesei*. To highlight differences in culture and anamorph morphologies, a short description of *H. jecorina*/*T. reesei* cultured in the same experiments is given here (Table 3). On CMD, mycelium covering the plate within 3 to 4 days at 25 to 37°C. Conidiation after 4 to 8 days at 25°C on CMD and SNA concentrated in a distal concentric zone, farinose, green; only shrubs, no well-defined pustules formed. Conidiophores

straight, 2.5 to 5 μm wide, similar to those of *T. parareesei* in shrubs. Larger branches becoming coarsely warted with age. Phialides solitary, lageniform, straight, only rarely slightly curved, sometimes with long cylindrical necks. Conidia variable, ellipsoidal, or oblong with parallel sides, scar indistinct.

Temperature-dependent growth of *T. parareesei* and *H. jecorina* on multiple carbon sources. We have recently reported that *T. parareesei* and *H. jecorina* exhibit qualitatively similar carbon source utilization profiles under standard temperature conditions (25°C) but respond differently to light (4). Moreover, it was noticed that *T. parareesei* showed a more variable utilization profile compared to that of *H. jecorina*. Here we performed phenotype microarrays (PMs) of both species at seven temperatures in darkness in order to get an advanced profile of carbon source utilization by *T. parareesei* in comparison to that of *H. jecorina*. Results show that in general both species have their growth optimum at temperatures between 28 and 35°C. However, *T. parareesei* is able to produce statistically higher biomass in this temperature range compared to that for *H. jecorina* (analysis of variance [ANOVA], $P < 0.05$). Figure 2A shows the temperature-dependent growth rates of both species calculated for the 16 best carbon sources (cluster I) for *H. jecorina*, as estimated by Druzhinina et al. (2). The detailed PM profiles are given in Table S1 in the supplemental material. The most distinct difference between PM profiles of the two species was detected at 35°C. Figure 2B shows examples of faster growth of *T. parareesei* at 35°C on *i*-erythritol, D-mannitol, D-cellobiose, and D-galactose than the rate for *H. jecorina*. An inspection of the growth pattern on *i*-erythritol and D-mannitol can be used in the laboratory to distinguish the two species. In addition to the carbon sources listed above, similar patterns were also observed on gentobiose, D-mannose, D-xylose, D-arabitol, D-trehalose, α-D-glucose, and D-fructose, yet without statistical significance (ANOVA, $P > 0.05$).

Phytotoxicity assays. We have previously reported that *T. parareesei* is considerably different from *H. jecorina* with respect to its ecophysiological adaptation (4). Thus, we found that, contrary to *H. jecorina*, *T. parareesei* is better adapted to growth in light and is more competitive with epigeal fungi than *H. jecorina*. However, all strains of *T. parareesei* have been isolated from soil, suggesting that soil may be its natural habitat. In order to extend our knowledge on the ecology of *T. parareesei*, we have performed a pilot phytotoxicity assay, which tests the influences of *T. parareesei* and *H. jecorina* on the germination of seeds and on the growth of a model plant, *Lepidium sativum*. This plant was chosen for its rapid growth in soil-free cultivations. The results show that both *T. parareesei* and *H. jecorina* are, in fact, able to inhibit growth of *L. sativum* up to 40%, yet the plants were normally developed. Statistically supported differentiation was detected only between each of the investigated fungal species and the control samples (for *T. parareesei*, $P = 0.0009$ [ANOVA] and $n = 59$; for *H. jecorina*, $P = 0.0002$ and $n = 67$), but no statistically significant differences were found between the species. Furthermore, the variation of inhibition among the *T. parareesei* strains was minimal. In contrast, growth of the plants with *H. jecorina* was strain dependent; in particular, strain C.P.K. 3419 inhibited *L. sativum* plants the most conspicuously. Development of the rootlets in the presence of *Trichoderma* was observed to be strain specific, as some strains (C.P.K. 717 and C.P.K. 667 of *T. parareesei* and C.P.K. 3419 of *H. jecorina*) clearly inhibited maturation of *Lepidium* roots.

Evolution of *T. parareesei*. The genealogical concordance phylogenetic species recognition criterion, based on three of the most polymorphic phylogenetic markers applied previously (4), showed that *T. parareesei* constitutes a cryptic phylogenetic species genetically isolated from *H. jecorina*. In order to learn about the evolution of these two species with respect to other related taxa, we used Bayesian phylogenetic analysis of nucleotide sequences of two relatively conserved phylogenetic markers, *rpb2* and *chi18-5*, which allow alignments with other species of *Trichoderma* section *Longibrachiatum* (Fig. 3). The nucleotide properties of these loci and parameters of phylogenetic analyses are given in Table 2. The topology of the phylogram based on the combined data set (Fig. 3A) confirms the result of Samuels et al. (20) and shows that, indeed, *T. longibrachiatum* and species related to it (*H. orientalis* and *Trichoderma* sp. strain C.P.K. 1837 [3]) are the next genetic neighbors to the species studied in this work. Furthermore, it also revealed a monophyletic Reesei subclade combining *T. parareesei*, *Trichoderma* sp. strain C.P.K. 524, and *H. jecorina* (Fig. 3B). The Reesei subclade is characterized by relatively short intercladal genetic distances compared to those present between other species in the section. A detailed inspection of this subclade (Fig. 3B) revealed that *T. parareesei* does not occupy any separate clade but is most closely related to the hypothetical common ancestor of the whole Reesei subclade. This was also confirmed on both individual phylograms for *rpb2* and *chi18-5* (data not shown). Three strains of *T. parareesei* (C.P.K. 3692 [Ethiopia], C.P.K. 634 [Ghana], C.P.K. 3426 [Sri Lanka]) are closest to the ancestral node, while four other strains form a statistically supported subclade derived from it. The phylogram shows that both *H. jecorina* and *Trichoderma* sp. strain C.P.K. 524 derived from the ancestral state, which is contemporarily represented by *T. parareesei*. In contrast to *T. parareesei*, strains of *H. jecorina* show considerable infraspecific polymorphism and occupy the longest branches of the Reesei subclade. These data indicate that *T. parareesei* is evolutionarily older than *H. jecorina* and likely resembles the ancestor of the latter species.

DISCUSSION

In this study we extend our earlier finding (4) of *T. parareesei* as a new phylogenetic species of *Trichoderma* section *Longibrachiatum* to a full taxonomic description. The use of an integrated approach consisting of morphological observation and description, temperature-dependent carbon source utilization profiling, and phylogenetic analysis resulted in a clear differentiation between *T. parareesei* and *T. reesei*, the anamorph of *H. jecorina*, where *T. parareesei* was erroneously ascribed to earlier (1, 7, 12).

Macro- and micromorphological characters. Conidiation in *T. parareesei* is distinctly more abundant than in *H. jecorina* on all media at all temperatures. On richer media such as PDA and MEA, conidiation in *T. parareesei* is conspicuously abundant and is positively correlated with temperatures up to 37°C. Under such conditions, several generations of bright yellow-green pustules form consecutive superposed layers, resulting in a thick, coarsely tubercular colony surface. In *H. jecorina*,

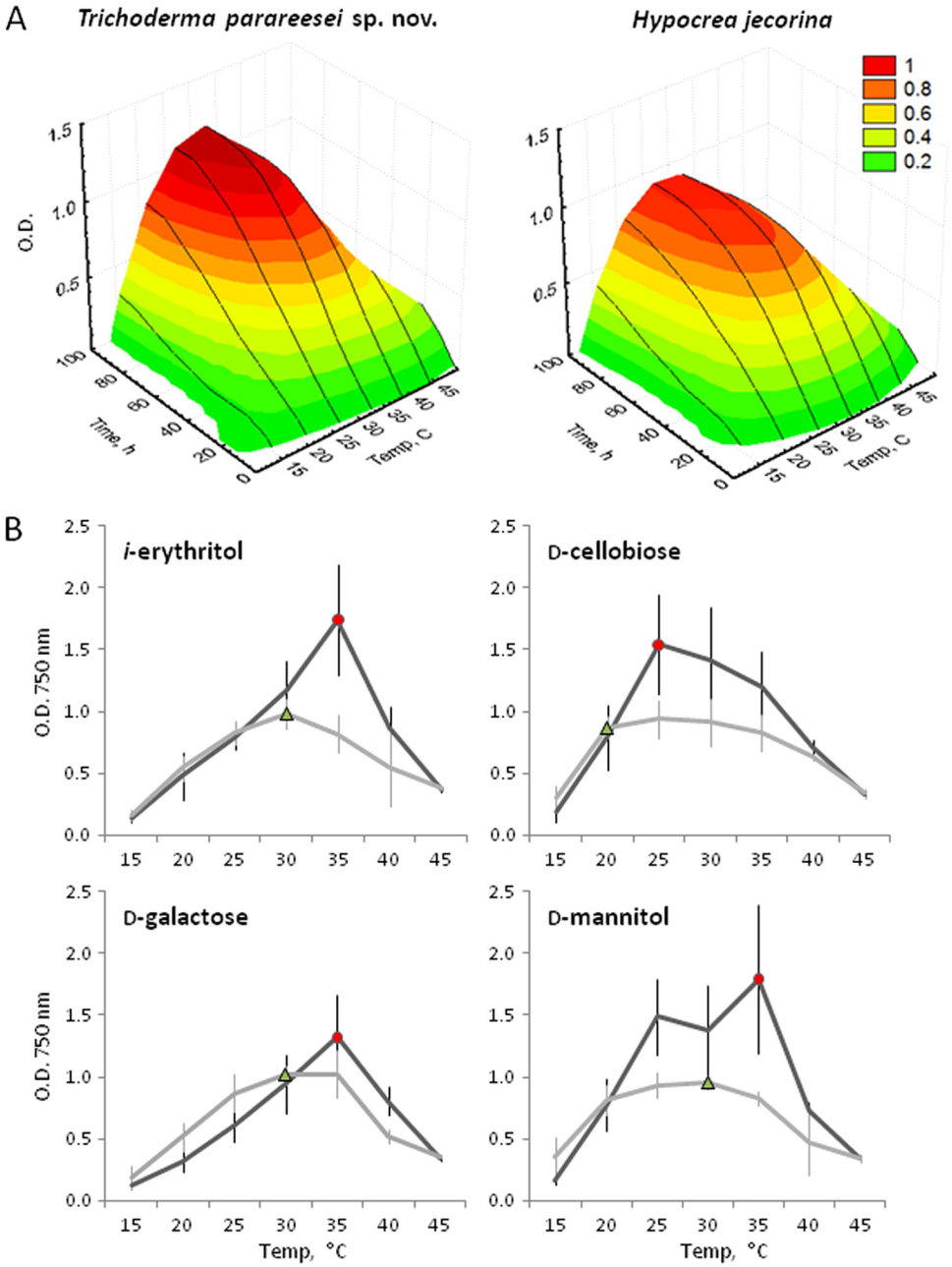

FIG. 2. Temperature-dependent growth of *T. parareesei* and *H. jecorina* on different carbon sources. (A) Temperature-dependent growth rates of both species calculated for the 16 best carbon sources (cluster I, L-arabinose, D-arabitol, D-cellobiose, dextrin, *i*-erythritol, D-fructose, gentobiose, α-D-glucose, maltotriose, D-mannitol, D-mannose, D-melezitose, D-trehalose, D-xylose, γ-aminobutyric acid), as estimated by Druzhinina et al. (2) for *H. jecorina.* The complete carbon source utilization profiles are given in Table S1 in the supplemental material. (B) Temperature-dependent growth of *T. parareesei* and *H. jecorina* on individual carbon sources. Vertical bars correspond to standard deviations, calculated on the basis of the profiles of six and five strains for *T. parareesei* and *H. jecorina*, respectively. Colored labels highlight the differences.

conidiation on PDA is usually yellow and turns green only slowly, while on CMD and SNA, only shrubs and no well-defined pustules are formed. Conidiophores of *H. jecorina* are comparable to those of *T. parareesei* and are formed in shrubs, but they are generally straighter, with slightly longer phialides and conidia that vary in shape and size more conspicuously than those of *T. parareesei*. Phialide length only rarely exceeds 10 μm in *T. parareesei*, while this is common in *H. jecorina*. The formation of a yellow pigment is variable among isolates of both species.

Reasons for genetic isolation and speciation. Our data show that *T. parareesei*, a species that has lost its ability to reproduce sexually, likely closely resembles the anamorphic stage of a common ancestor of all three species detected in the Reesei subclade. We have recently demonstrated that although *T. parareesei* possesses both the *MAT1-1* and *MAT1-2* loci, the

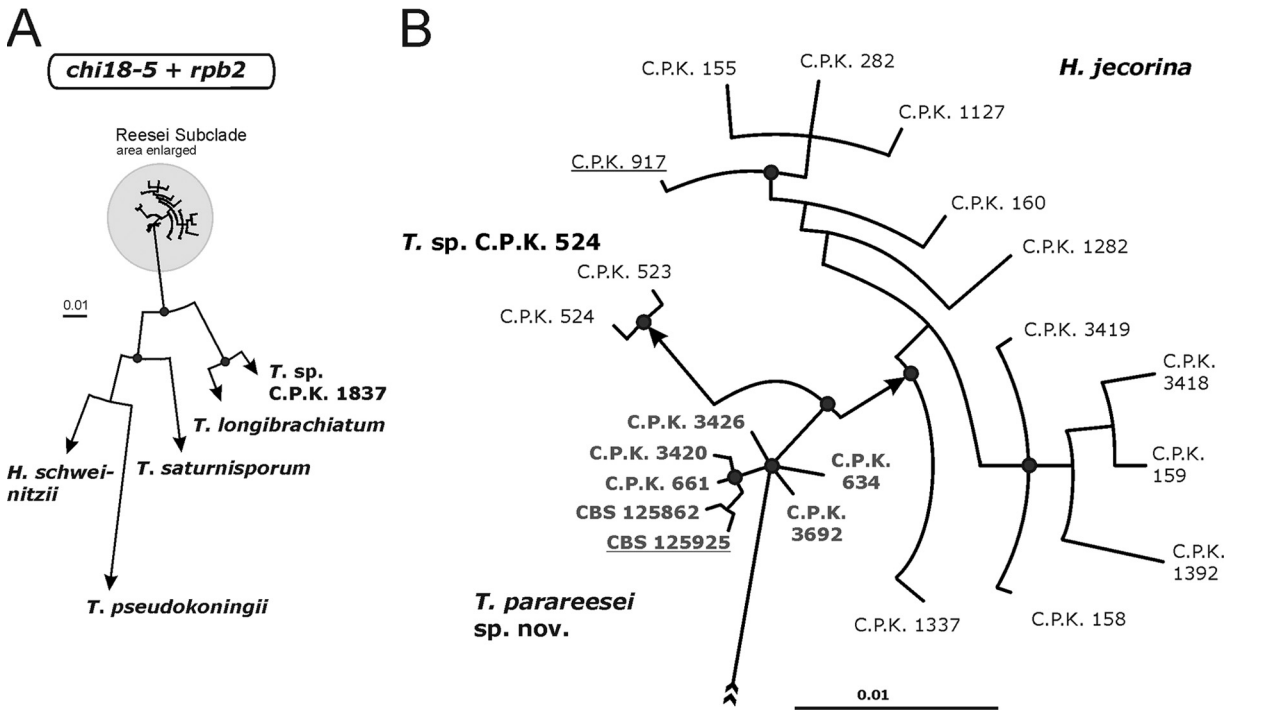

FIG. 3. Bayesian circular phylogram inferred from the concatenated data set of partial exons of *rpb2* and *chi18-5* phylogenetic markers. Symbols at the nodes correspond to PPs of >94%. Arrows point to clades/nodes of phylogenetic species. (A) Phylogenetic position of the Reesei subclade with respect to other species from *Trichoderma* section *Longibrachiatum*; (B) phylogenetic relations between species of the Reesei subclade (enlarged from panel A). The ex type strain of *T. reesei* QM 6a (C.P.K. 917) is underlined.

latter is essentially altered compared to that of *H. jecorina*, where both mating-type loci are functional even *in vitro*. Therefore, we speculate that the inability of sexual reproduction likely originated from mutations in the *MAT1-2* gene (4). Here we used conservative phylogenetic markers (coding fragments of the *chi18-5* and *rpb2* genes) to infer the evolution of the group of species rather than to differentiate *T. parareesei* from the rest. The analyses show that, in fact, *T. parareesei* is the oldest taxon which apparently nearly stopped its evolutionary development and that *H. jecorina* and *Trichoderma* sp. strain C.P.K. 524 arose from it. This is in perfect agreement with our previous results, which showed abundant molecular footprints of sexual recombination for *H. jecorina* and none for *T. parareesei* (4). Thus, we conclude that *T. parareesei* is a relict agamospecies which resembles the ancestor of *H. jecorina* and *Trichoderma* sp. strain C.P.K. 524. In this case, it is interesting to speculate on reasons which led to the survival of *T. parareesei*. The reduction of sexual recombination is reflected in the low level of infraspecific polymorphism of *T. parareesei*, which theoretically makes the species vulnerable to changing environments. We think that the survival of *T. parareesei* was to a large extent possible due to the long-term stability of its habitat, the tropical forest, which is one of the most sustainable and ancient ecosystems on the planet. However, we have also observed that *T. parareesei* has a versatile phenotype in a broad range of temperatures, extensive conidiation, and fast growth rates, which altogether are characteristics in line with strongly opportunistic members of the genus, e.g., *T. asperellum*, *T. longibrachiatum*, and *T. hamatum.* As it is hard to imagine which mechanisms could be exploited by the agamospecies to gain new genetic/phenetic properties (6), we would rather suggest that in *H. jecorina* extensive production of propagules and growth on certain carbon sources was reduced during evolution. This hypothesis is well supported by the observations that *H. jecorina* has an unusual small genome compared to the sizes of the genomes of other ascomycetes and *Trichoderma* species (16; cf. also http://genome.jgi-psf.org/Trive1/Trive1.home.html and http://genome.jgi-psf.org/Triat1/Triat1.home.html), suggesting that its overall phenotype likely reflects some losses in the genome. Druzhinina et al. (4) also showed that *H. jecorina* is photoinhibited to a certain extent, while *T. parareesei* is well adapted to various lighting conditions. Moreover the two species also have differences in their antagonistic potential in dual confrontations with fungi pathogenic for plants, showing that *T. parareesei* is generally more competitive. These considerations call for a comparison of the genomes of *T. parareesei* and *H. jecorina*, because this may provide insights into the original genetic potential of this important industrial species, where speciation occurred by restriction to a narrow habitat and lifestyle.

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